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Generation of a comprehensive panel of crustacean allergens from the North Sea Shrimp *Crangon crangon*

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ABSTRACT

Background: Published data on crustacean allergens are incomplete. The identification of tropomyosin (TM), arginine kinase (AK), sarcoplasmic Ca-binding protein (SCP) and myosin light chain (MLC) as shrimp allergens are all important contributions but additional allergens are required for the development of a complete set of reagents for component resolved diagnosis and the exploration of novel vaccination strategies.
Methods: The North Sea shrimp (*Crangon crangon*), which is frequently consumed in Europe, served as a model organism in this study. TM and AK were directly cloned from mRNA based on sequence homology and produced as recombinant proteins. Additional IgE-reactive proteins were isolated by preparative SDS-PAGE and identified by mass spectrometry and corresponding cDNAs were cloned and expressed in *E. coli*. The relevance of the 6 cloned crustacean allergens was confirmed with sera of 31 shrimp-allergic subjects, 12 of which had a positive double-blind, placebo-controlled food challenge (DBPCFC) to shrimp and 19 a convincing history of food allergy to shrimp, including 5 cases of anaphylaxis. Quantitative IgE measurements were performed by ImmunoCAP.
Results: Six recombinant crustacean proteins: TM, AK, SCP, a novel MLC, troponin C (TnC), and triosephosphate isomerase (TIM) bound IgE in ImmunoCAP analysis. Specific IgE to at least one of these single shrimp allergens was detected in 90% of the study population, thus the *in vitro* diagnostic sensitivity was comparable to that of shrimp extract (97%). In 75% of the subjects, the combined technical sensitivity was similar to or greater with single shrimp allergens than with natural shrimp extract.
Conclusions: We identified six IgE-binding proteins from *C. crangon*, three of which have not before been described as allergens in crustaceans. This extensive panel of shrimp allergens forms a valuable asset for future efforts towards the identification of clinically relevant biomarkers and as a basis to approach patient-tailored immunotherapeutic strategies.

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1. Introduction

There is limited knowledge regarding the crustacean proteins responsible for eliciting allergic reactions. Only four proteins have so far been identified as crustacean allergens. At first, the muscle protein tropomyosin was reported to be a major allergen in various crustacean species (reviewed by Lehrer et al., 2003; Motoyama et al., 2007, 2008; Nakano et al., 2008) but it was also identified as IgE-binding in other arthropods such as cockroaches (Jeong et al., 2003; Sookrung et al., 2009) mites (Aki et al., 1995; Asturias et al., 1998) as well as mollusks (Asturias et al., 2002; Miyazawa et al., 1996). Thus tropomyosins would seem to be involved in cross-reactivity among different invertebrates (reviewed by (Lopata and Lehrer, 2009)). Allergens of a second protein family, arginine kinases, were described from crustaceans (Garcia-Orozco et al., 2007; Yu et al., 2003), insects (Binder et al., 2001; Sookrung et al., 2006), as well as arachnids (Hales et al., 2007).

Abbreviations: AK, arginine kinase; FPLC, fast protein liquid chromatography; IMAC, immobilized metal affinity chromatography; LC, liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MLC, myosin light chain; MS, mass spectrometry; SCP, sarcoplasmic Ca-binding protein; TIM, triosephosphate isomerase; TM, tropomyosin; TnC, troponin C.

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Even though tropomyosin has been reported to be the most important allergen for patients from the USA and Asia (Daul et al., 1994), little is known about the sensitization pattern in European subjects. Recently, sarcoplasmic Ca-binding proteins from black tiger shrimp (*Penaeus monodon*) (Pen m 4) (Shiomi et al., 2008) and white Pacific shrimp (*Litopenaeus vannamei*) (Lit v 4) (Ayuso et al., 2009), and the myosin light chain from *L. vannamei* (Lit v 3) (Ayuso et al., 2008) were described as novel shrimp allergens, the latter being a homologue of Bla g 8 from German cockroach (*Blatella germanica*). However, additional IgE-binding proteins can be detected in extract immunoblots (Ayuso et al., 2008; Daul et al., 1994) and it is suggested that the four proteins identified so far do not represent a complete panel of shrimp allergens.

Certain allergenic proteins might be underrepresented in diagnostic or therapeutic allergen extracts due to low concentration in the source material or unfavorable conditions during extract preparation. As has been demonstrated for several allergenic foods, the use of both major and minor single allergen components may increase the diagnostic sensitivity of component-resolved diagnosis (CRD), and sometimes reveals correlations between IgE reactivity to certain components and the clinical status of the patients (Bauermeister et al., 2009; Bublin et al., 2010; Reuter et al., 2006). Additionally, it is an interesting observation that the designation as minor and major allergens are not necessarily absolute; for example, non-specific lipid transfer proteins (nsLTP) are of major importance in plant food allergy in southern Europe (Scheurer et al., 2001; Schocker et al., 2004) whereas clinically relevant nsLTP sensitization is rarely found in northern Europe.

This study aimed at creating a more complete understanding of relevant shrimp allergens, in part for the purpose of enabling a more extensive component-resolved diagnostic study on shrimp allergy within the EU-funded multi center study EuroPrevall. Detailed knowledge on the full repertoire of IgE-binding proteins will also be helpful towards the understanding of cross-reactivities affecting the management of seafood-allergic patients and the development of improved diagnostic and immunotherapeutic reagents.

As the study investigates IgE binding in European subjects the North Sea shrimp *Crangon crangon*, which has a significant commercial relevance in Europe was chosen as a model organism.

This study describes the generation of a comprehensive panel of *C. crangon* allergens and the IgE-binding diversity to these allergens among 31 shrimp-allergic subjects from Europe.

2. Material and methods

2.1. Sera

As serum samples of well characterized shrimp-allergic subjects were available in limited amounts, the initial assessment of novel allergen candidates was performed with plasma samples of eight North American subjects having increased shrimp specific serum IgE but unknown clinical history (Table 1, sera A-H: PlasmaLab International, Everett, WA, USA). Allergen candidates were then evaluated with sera of 31 subjects with confirmed shrimp allergy, 17 of which were recruited at the University Hospital Zurich (Zurich, Switzerland) (Table 1, sera 1–17). Their shrimp allergy was confirmed by either a positive double-blind, placebo-controlled food challenge (sera 1–12, the study was approved by the local ethical committee of the University of Zürich) or a clear history of anaphylaxis to shrimp (sera 13–17). Fourteen subjects were included based on a clear history of an allergic reaction after ingestion of shrimp, thereof three from Spain (Table 1, sera 18–20: Hospital General de Castellón, Castellón) and eleven from Germany (Table 1, serum 21: serum bank of the Paul-Ehrlich-Institut, sera 22–31: DLab Diagnose GmbH, Hamburg). The most relevant clinical patient data and levels of shrimp specific IgE, measured by ImmunoCAP (Phadia, Uppsala, Sweden), are summarized in Table 1. All subjects provided written informed consent.

Table 1
Clinical characteristics of 31 shrimp-allergic subjects (1–31) and 8 subjects with sensitization to shrimp (A-H), including specific IgE values to shrimp extract.

Serum	Sex	Age	SPT shrimp	Symptoms	Specific IgE to shrimp extract determined by ImmunoCAP (f24)
1	m	27	+	U, AE, E, D	81.50
2	m	30	–	E, N	1.45
3	m	35	–	U, R, LE, V, U (contact)	6.13
4	m	61	+	AE, N, R, LE, OAS	3.00
5	f	33	–	E, N	<0.1
6	m	67	+	AE, E, D, N, OAS	0.50
7	m	51	+	U, LE, OAS, BD	2.55
8	m	32	+	U, E, OAS	25.30
9	m	Nd	+	E, N, GIT, Dr	0.66
10	m	36	+	OAS	0.76
11	f	22	+	D	1.04
12	m	23	+	AE, OAS	16.30
13	m	27	+	severe D	13.80
14	f	40	+	U, AE, D, BD	1.19
15	f	27	+	AE, D, LE	>100
16	m	22	+	severe D	18.20
17	f	43	+	U, N, BD	7.22
18	m	35	+	U, AE	0.25
19	f	34	+	U, AE	0.81
20	m	18	+	U	3.39
21	m	54	nd	AE, D	>100
22	f	40	+	BD	1.41
23	f	47	+	AE, LE	0.77
24	f	42	+	BD	1.35
25	f	41	+	V, BD, D	18.35
26	f	54	+	P	2.13
27	f	69	–	AE, D	3.24
28	f	46	+	P, U, AE, BD, D	14.65
29	f	52	+	AE, U	2.53
30	m	47	+	AE, U	14.94
31	f	18	+	AE	33.87
A	m	35	nd	nd	8.22
B	f	38	nd	nd	3.31
C	m	32	nd	nd	5.01
D	nd	52	nd	nd	14.80
E	nd	Nd	nd	nd	4.65
F	m	12	nd	nd	2.51
G	m	33	nd	nd	54.10
H	m	19	nd	nd	13.50

f: female, m: male, U: urticaria, AE: angioedema, E: emesis, D: dyspnea, N: nausea, R: rhinitis, LE: laryngeal edema, V: vertigo, OAS: oral allergy syndrome, BD: hypotension, GIT: gastrointestinal problems, Dr: diarrhea, P: pruritus, nd: unknown/not determined.

2.2. Food challenge procedure

Double-blind, placebo-controlled oral shrimp challenges were conducted on 12 subjects from University Hospital Zurich. The challenge consisted of increasing doses (10 μg, 100 μg, 1 mg, 10 mg, 100 mg, 1 g and 4 g) of cooked shrimp concealed in a 20 g (after cooking) seasoned, cooked ground beef patty (Taylor et al., in preparation). Three placebos (seasoned ground beef patty to which no shrimp was added) were randomly interspersed in the group of shrimp challenge doses. Challenges were given at 30 min intervals for the 10 μg–10 mg doses and 45 min intervals for the 100 mg–4 g doses. Neither the test subject nor the administering clinician had knowledge of the placebo placement. Subjects who had not reacted in an objective fashion after the blinded challenges were given open challenges with 4 g and then 16 g at one hour intervals to confirm reactivity to shrimp.

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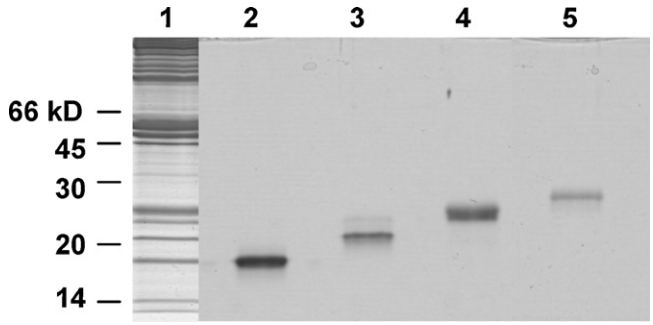

Fig. 1. Coomassie Brilliant Blue staining of *C. crangon* extract and protein bands purified by preparative SDS-PAGE, lane 1-IEF buffer extract from *C. crangon*, lane 2–18 kDa fraction, lane 3–21 kDa fraction, lane 4–24 kDa fraction, lane 5–28 kDa fraction.

2.3. Fractionation of natural IgE-reactive C. crangon proteins by preparative SDS-PAGE

Cooked shrimp tail meat (*C. crangon*) was purchased locally (Frankfurt, Germany), frozen in liquid nitrogen, ground, precipitated with −20 °C cold acetone, extracted with a buffer containing high concentrations of urea (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris, 65 mM DTT, 0.2% Biolyte 3–10 (BioRad, Hercules, CA, USA)), and clarified by centrifugation (1.5 h, 15,000 × *g*). Low molecular weight allergens were purified by preparative SDS-PAGE (Model 491 PrepCell, BioRad) as described previously (Reese et al., 2006). Briefly, *C. crangon* proteins were separated on a 28 mm-wide vertical column containing a 15 mm stacking gel (5%T, 1.5%C) on top of a 65 mm separation gel (17.5%T, 1.5%C), using Laemmli's discontinuous SDS-PAGE buffer system (Laemmli, 1970). Fractions containing IgE-reactive *C. crangon* proteins were identified by immunoblot analysis with selected sera, pooled, and dialyzed against deionized water. Purity was approximately 95%, as judged by analytical SDS-PAGE and Coomassie Brilliant Blue staining (Fig. 1).

2.4. Identification of putative crustacean allergens by mass spectrometry and de novo sequencing

In-gel digestion of protein bands excised from SDS-PAGE gels was performed according to Shevchenko (Shevchenko et al., 1996) with several modifications (Albrecht et al., 2008). Tryptic digests were separated on a nano-HPLC (1100 series, Agilent Technologies, Santa Clara, CA, USA; column: zorbax-300SB-C18, 150 mm × 75 μm, gradient: water containing 0,1% formic acid (FA) to 55% acetonitrile plus 0.1% FA in 40 min at 300 nL/min) and analyzed with a nano-ESI IonTrap mass spectrometer that was set to standard parameters (MSD-Trap-XCT plus, Agilent). Searches against the entire NCBI database were performed using Mascot. The search parameters were as follows: fixed modifications: carbamidomethyl (C), variable modifications: oxidation (M), protein mass: unrestricted, peptide mass tolerance: ±1.2 Da, fragment mass tolerance: ±0.6 Da, max missed cleavages: 1. Hits were accepted at a probability value of $p < 0.05$. *De novo* sequencing was performed by manual spectrum interpretation.

2.5. Cloning of C. crangon cDNAs

Total RNA was isolated from pulverised, snap-frozen live *C. crangon* using TRIZOL reagent (Life Technologies, Eggenstein, Germany) according to the manufacturer's instructions. First strand cDNA was generated by the First Strand cDNA Synthesis-Kit (Amersham Bioscience, Freiburg, Germany). The 3′ ends were cloned with target-specific primers that were selected on the basis of published sequences of homologous proteins from different crustaceans. The 5′ ends were cloned with the 5′RACE System for Rapid Amplification of cDNA ends (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The full-length cDNA sequences were amplified with gene-specific primers (Table 2).

Table 2
Gene-specific primer sequences used for amplification of cDNA from *C. crangon* which was used for expression of tropomyosin (TM), arginine kinase (AK), sarcoplasmic Ca-binding protein (SCP), myosin light chain (MLC), troponin C (TnC), and triosephosphate isomerase (TIM).

Protein	Orientation	Primer sequence
TM	forward	5′-GCCATATGGACGCCATCAAGAAGAAGATG-3′
	reverse	5′-GAGCGGCCGCGTAGCCAGACAGTTCGCTGAAAG-3′
AK	forward	5′-GACCATATGGTTGATGCAGAAGTTCTG-3′
	reverse	5′-TCGCTCGAGCATCTCCTTCTCCATCTTG-3′
SCP	forward	5′-ATATATCATATGGCTTACACTTGGGATAACCG-3′
	reverse	5′-TATATACTCGAGAACGACTTCCTTGAGGGGGC-3′
TnC	forward	5′-ATATATCATATGGATTCACTTGAGCCTGACCA-3′
	reverse	5′-TATATACTCGAGACCGCTCATCATCTCCATGAATT-3′
MLC	forward	5′-ATATATCATATGGCCGCGGATCTTAGTGC-3′
	reverse	5′-TATATACTCGAGAAGCAGCTGCGTCAACTTCTTG-3′
TIM	forward	5′-ATATATCATATGTCGGGTTCAAGGAAGTTTTT-3′
	reverse	5′-TATATACTCGAGACCCCGGGCATTAATAATGG-3′

2.6. Expression and purification of recombinant C. crangon proteins

Allergen-encoding cDNAs were cloned into the expression vector pET23b (Novagen, Madison, WI, USA). The constructs were transformed into an *E. coli* BL21 strain (Novagen, ordered through VWR International, Darmstadt, Germany) which carried the plasmid pT7POL23 (Belgian Coordinated Collections of Microorganisms (BCCM), Ghent, Belgium) (DeWitt et al., 2004; Mertens et al., 1995). pT7POL23 provides for heat-inducible, tightly controlled expression of the T7 RNA polymerase. Cells were grown in Luria Broth at 28 °C. The temperature was increased to 42 °C to induce protein expression when $OD_{600\,nm}$ had reached 0.6. After 4 h cells were harvested by centrifugation at 5000 × *g* at 4 °C and resuspended in lysis buffer (50 mM NaH_2PO_4, 500 mM NaCl, 2 mM imidazole, pH 8.0). The suspension was stored at −80 °C over night. After thawing, lysozyme and EDTA-free protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) were added to the solution and shaken for 15 min at room temperature. Afterwards 0.2% Triton, 2 mM $MgCl_2$ and 10 μL benzonase (Merck KGaA, Darmstadt, Germany) were added, followed by three sonication cycles (settings: power 60%, pulse 15 s, break 15 s) and clarification by centrifugation at 7500 × *g* and 4 °C. For proteins (TM, AK, SCP, and MLC) insoluble in lysis buffer, pellets were resuspended in lysis buffer containing 6 M urea and clarified by centrifugation. Cell lysates containing the major protein portion were loaded on a Ni-NTA resin (His-Gravi Trap™, GE Healthcare, Freiburg, Germany). $(His)_6$-tagged proteins were eluted with increasing amounts of imidazole. TnC was additionally purified by gel filtration on a Superdex 75 prepgrade 16/60 column, run with an FPLC instrument (Amersham Pharmacia Biotech, Freiburg, Germany). Protein concentrations were determined using the PIERCE BCA Protein Assay (Perbio Science, Bonn, Germany), with BSA as protein standard.

2.7. CD spectroscopy

CD spectra were recorded from purified recombinant proteins diluted in 10 mM potassium phosphate buffer (pH 7.0) in a quartz cuvette (Hellma, Müllheim, Germany) with a path length of 1 mm using a Jasco J-810 S spectropolarimeter (Jasco, Groß-Umstadt,

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Germany). The measurement parameters were: 20 °C temperature, 1 nm band width, 0.2 nm step width, 190–250 nm spectral range and 50 nm/min scanning speed. Ten scans were accumulated to increase the signal-to-noise-ratio.

2.8. MALDI-TOF MS analysis of recombinant C. crangon allergens

Protein bands were excised from Coomassie Brilliant Blue stained SDS-PAGE gels, digested with trypsin and the resulting peptides were analysed by MALDI-TOF MS as described elsewhere (Albrecht et al., 2008).

2.9. Immunoblot analysis

SDS-PAGE was performed with 12.5%T, 2.67%C separating gels and 5%T, 2.67%C stacking gels using a discontinuous buffer system (Laemmli, 1970). For immunoblot analysis, electrophoretically separated proteins (0.5 μg/cm of recombinant protein or 20 μg/cm of total protein extract) were transferred onto nitrocellulose membranes by semi-dry blotting at 0.8 mA/cm^2 for 45 min (Kyhse-Andersen, 1984). After blocking with Tris-buffered saline (TBS) containing 0.3% Tween 20 blots were cut into strips and incubated over night at room temperature with subjects' sera, diluted 1:12 with TBS containing 0.05% Tween 20 (TBST) and 0.1% BSA (TBST-BSA). After incubation for 1 h with alkaline phosphatase-conjugated, monoclonal mouse anti-human IgE antibody (G7-26 hybridoma, BD Bioscience, Franklin Lakes, NJ, USA), diluted 1:750 with TBST-BSA the IgE-binding proteins were visualized by chemiluminescence (Tropix CDP Star® detection system, Applied Biosystems, Bedford, MD, USA) or by chromogenic staining (mixture containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT, Sigma, St. Louis, MO, USA) dissolved in 100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) (Leary et al., 1983).

In order to obtain objective results from immunoblot analysis of recombinant shrimp proteins, intensities of IgE-binding were recorded by densitometry using the Lumi Imager and the Lumi-Analyst v3.1 software (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The Cut-off level was defined as the fourfold signal intensity of the non-allergic control. When required elevated background signals were subtracted manually.

2.10. Determination of specific IgE levels

Purified recombinant allergens from *C. crangon* as described above were coupled to ImmunoCAP solid phase as described (Marknell DeWitt et al., 2002). For measurement of IgE to natural shrimp extract, commercial ImmunoCAP tests were used.

Specific IgE antibodies to shrimp extract and the newly identified allergens from *C. crangon* were measured by the ImmunoCAP system (ImmunoCAP 250 assay instrument, Phadia, Uppsala, Sweden) according to the manufacturer's instructions. Concentrations of specific IgE ≥ 0.1 kU_A/L were considered positive.

3. Results

3.1. Strategy for identification of IgE-reactive proteins from the North Sea Shrimp C. crangon

Previous studies (Ayuso et al., 2008; Daul et al., 1994) suggested the presence of IgE-reactive proteins in shrimp extracts additional to the already known allergens tropomyosin and arginine kinase. We therefore aimed at identifying enriched IgE-binding protein fractions by means of a preparative SDS-PAGE followed by IgE immunoblotting and mass spectrometry. Four IgE-reactive shrimp

Table 3
Proteins identified by MS/MS database search (protein bands at 21 kDa, 24 kDa and 28 kDa) and *de novo* sequencing (protein band at 18 kDa) as potential novel crustacean allergens.

Fraction	Peptide	Matching identity	Species of origin	GenBank accession no.	Amino acid positions
18 kDa	NLNPTLALIAK	Myosin light chain alkali (MLC)	Artemia franciscana (brine shrimp)	ABS19977	27–37
	DFMPI(F/M_{ox})AQVK				53–62
21 kDa	FLIEDEEALK	Troponin C (TnC)	Homarus americanus (American lobster)	P29291	75–85
	FLIEDEEALKAELR				75–89
	DYEINELNIQVNDLR	Troponin I	Pontastacus leptodactylus (narrow-clawed crayfish)	P05547	123–137
24 kDa	IMSNLWNEIAELADFNK	Sarcoplasmic calcium-binding protein (SCP)	Pontastacus leptodactylus (narrow-clawed crayfish)	P05946	56–72
	DGEVTIDEFK				73–82
	KAVQNVCVGK				83–92
	IMSNLWNEIAELADFNKDGEVTIDEFK				56–82
	LDCISR				127–132
	TFIANQFK	Sarcoplasmic calcium-binding protein (SCP)	Procambarus clarkii (red swamp crayfish)	ABB58783	104–111
	TIDVNGDGLVGVDEYR				112–127
	YMYDIDNDGFLDK	Sarcoplasmic calcium-binding protein (SCP)	Penaeus sp. (shrimp species)	P02635	14–26
	AGGINLAR				156–163
28 kDa	VGHALEAGLK	Triosephosphate isomerase (TIM)	Archaeopotamobius sibiriensis (crayfish species)	CAD29196	104–113
	TEEVVFAQMK				130–139
	ALVPNISDWSR				140–150
	VVIAYEPVWAIGTGK				151–165

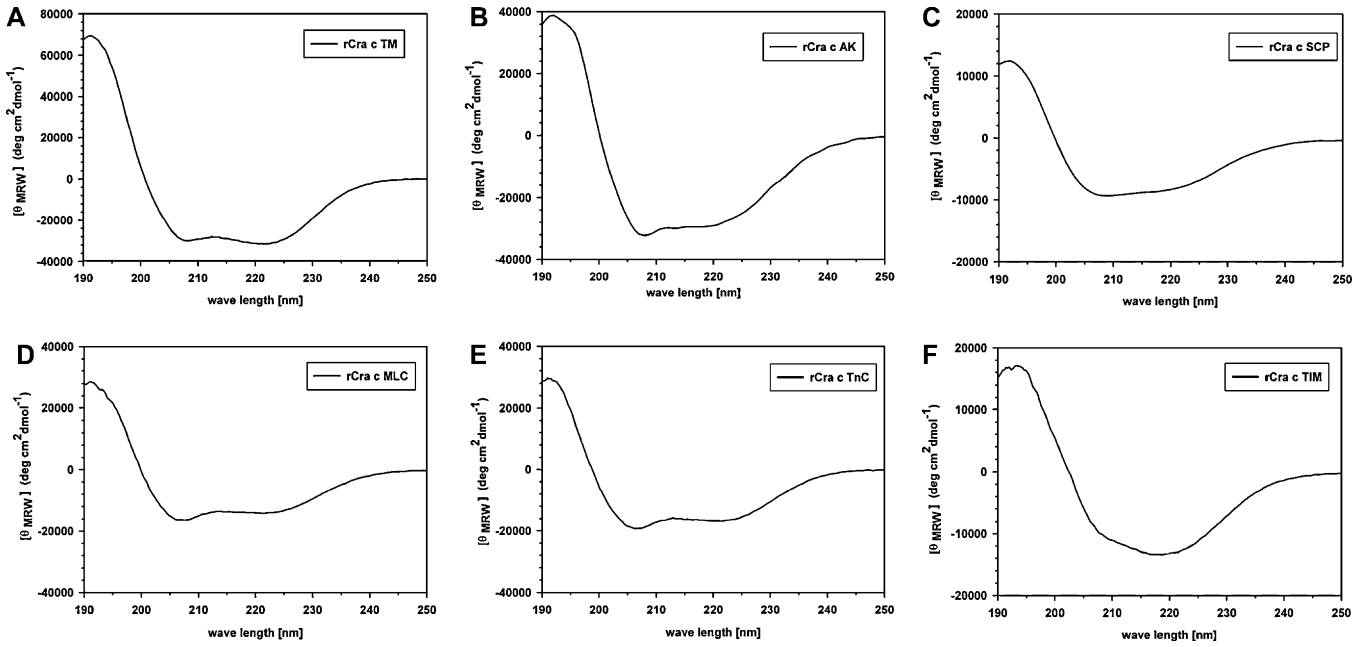

Fig. 2. CD-spectra of recombinant *C. crangon* allergens, (A) tropomyosin (TM), (B) arginine kinase (AK), (C) sarcoplasmic Ca-binding protein (SCP), (D) myosin light chain (MLC), (E) troponin C (TnC), (F) triosephosphate isomerase (TIM).

proteins, with apparent molecular weights of 18 kDa, 21 kDa, 24 kDa and 28 kDa, were isolated by preparative electrophoresis, at a purity of >95% as estimated by gel-electrophoresis (Fig. 1). In addition tropomyosin (TM) and arginine kinase (AK) were cloned from *C. crangon* by a PCR strategy utilizing available cDNA sequences of homologous allergens for primer design. Plasma samples from 8 shrimp sensitized donors were used for initial assessment of IgE reactivity of the shrimp proteins studied. Subsequently, their relevance and importance were evaluated with sera of patients ($n = 31$) with clinically confirmed allergy to shrimps.

3.2. Mass-spectrometry identified four C. crangon proteins as potential novel crustacean allergens

LC–tandem ESI-MS/MS analysis followed by a database search of the 21 kDa, 24 kDa and 28 kDa proteins identified them as troponin C (TnC), sarcoplasmic Ca-binding protein (SCP), and triosephosphate isomerase (TIM), respectively, of which TnC and TIM have not been previously identified as crustacean allergen (Table 3). One peptide of the 21 kDa protein also gave a match to troponin I in the database search, but as the match of two other peptides to troponin C was much more convincing, the troponin I hit was disregarded in the further analysis. *De novo* sequencing of peptides from the 18 kDa protein added the light chain of myosin (MLC) as an additional potential allergen.

3.3. Recombinant production and molecular characterization of six C. crangon allergen candidates

The complete open reading frames of TM, AK, SCP, MLC, TnC and TIM were cloned from *C. crangon* and used for expression in *E. coli* and purification of the corresponding recombinant proteins. cDNA and amino acid sequences of these six proteins are available and accession numbers are indicated in Table 4. The protein identities were confirmed by MALDI-TOF MS analysis of tryptic digests. In total 5–16 peptides were identified for each protein, resulting in a sequence coverage of 26–69% of the entire proteins (Table 5). Folding properties of the recombinant shrimp proteins were analyzed by CD spectroscopy. All six proteins gave rise to spectra consistent with the presence of distinct secondary structure elements (Fig. 2), with varying proportions of α-helices and β-sheets. These CD spectra correlated well with published CD spectra of homologous proteins from other species (data not shown).

Table 4
Novel *C. crangon* allergens – biological function, physicochemical characteristics, GenBank accession number, allergen nomenclature and sequence identities to homologous crustacean proteins.

Allergen	Biological function	Theoretical MW [kDa]	Theoretical pI	GenBank accession number	Allergen name	Sequence identity with crustacean proteins identified by MS/MS database search (Accesion Number, species of origin)
TM	Tropomyosin	32.8	4.7	FJ457621	Cra c 1	97.5% (AAZ76743. *P. aztecus*)
AK	Arginine kinase	40.1	5.8	FJ457622	Cra c 2	93.3% (AAO15713. *P. monodon*)
SCP	Sarcoplasmic Ca-binding protein	22.1	4.7	FJ462737	Cra c 4	82.4% (P05946. *P. leptodactylus*)
MLC	Myosin light chain	17.4	4.7	FJ462739	Cra c 5	52.5% (ABS19977. *A. franciscana*)
TnC	Troponin C	16.8	8.9	FJ462740	Cra c 6	78.0% (P29291. *H. americanus*)
TIM	Triosephosphate isomerase	27.0	5.5	FJ462738	Cra c 8	79.1% (CAD29196. *A. sibiriensis*)

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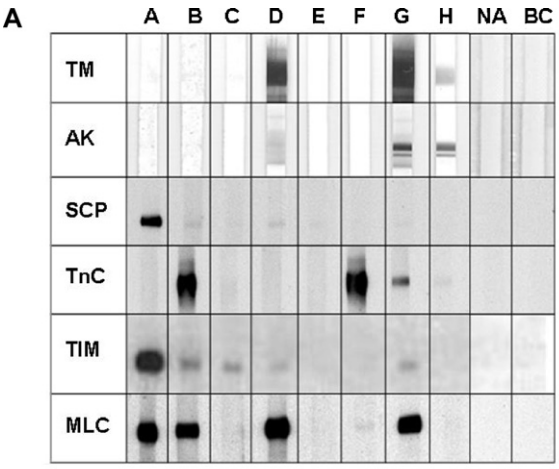
Table 5
Verification of identity of recombinant *C. crangon* allergens by MALDI-TOF MS: tropomyosin (TM), arginine kinase (AK), sarcoplasmic Ca-binding protein (SCP), myosin light chain (MLC), troponin C (TnC), and triosephosphate isomerase (TIM).

Allergen	Number of identified peptides	Sequence coverage
TM (Cra c 1)	16	56%
AK (Cra c 2)	8	26%
SCP (Cra c 4)	6	32%
MLC (Cra c 5)	5	43%
TnC (Cra c 6)	5	43%
TIM (Cra c 8)	12	69%

3.4. Immunoblot and ImmunoCAP analysis confirm IgE-binding of six C. crangon allergen candidates

The recombinant *C. crangon* proteins were initially screened for IgE reactivity with eight sera of shrimp sensitized subjects (sera A-H). All six proteins showed IgE-binding in immunoblot analysis (Fig. 3). After confirmation of their IgE reactivity, recombinant *C. crangon* proteins were used to prepare experimental ImmunoCAP tests for quantitative IgE determinations (Table 6). The IgE measurements on sera of the sensitized subjects were compared to the immunoblotting results and found to be in good correlation. All subjects with IgE reactivity in immunoblot analysis were also positive in ImmunoCAP testing. Additionally, detectable IgE was present in some sera for which no signal could be observed in immunoblot analysis, indicating that ImmunoCAP was the more sensitive of the two methods under the conditions used. The frequency of detectable IgE was 8/8 (100%) for AK and TIM, 7/8 (88%) for TM, TnC and MLC, and 6/8 (75%) for SCP. The non-atopic control was negative by both immunoblot and ImmunoCAP. Because of a higher sensitivity and an objective cut-off level for positive IgE-binding the ImmunoCAP assay was chosen for the analysis of sera of well-characterized shrimp-allergic subjects (Table 1, serum nos. 1–31).



B

	A	B	C	D	E	F	G	H	NA	BC	prevalence
TM	-	-	-	+	-	-	+	+	-	-	3/8 (38%)
AK	-	-	-	+	-	-	+	+	-	-	3/8 (38%)
SCP	+	+	-	+	-	-	-	-	-	-	3/8 (38%)
TnC	-	+	-	-	-	+	+	+	-	-	4/8 (50%)
TIM	+	+	+	+	-	-	+	-	-	-	5/8 (63%)
MLC	+	+	-	+	-	+	+	-	-	-	5/8 (63%)

Fig. 3. (A) IgE-binding in immunoblot analysis of 8 sera of shrimp sensitized subjects (A-H) and controls (NA: non-atopic subject, BC: buffer control) to purified recombinant *C. crangon* proteins tropomyosin (TM), arginine kinase (AK), sarcoplasmic Ca-binding protein (SCP), troponin C (TnC), triosephosphate isomerase (TIM) and myosin light chain (MLC). (B) Evaluation of positivity as obtained by cut-off based densitometric analysis (+: positive IgE-binding, –: no IgE-binding) and prevalence of IgE-binding at or above the cut-off level.

3.5. Confirmation and prevalence of IgE-binding to six C. crangon allergens

All six *C. crangon* proteins proved to be IgE-binding with sera of at least five allergic subjects (Table 7) and therefore fulfilled the criteria of the IUIS Allergen Nomenclature Subcommittee to be denominated allergens. The names that were assigned by the committee to TM, AK, SCP, MLC, TnC, and TIM of *C. crangon* are Cra c 1, Cra c 2, Cra c 4, Cra c 5, Cra c 6, and Cra c 8, respectively. Furthermore, the allergen sequence data were submitted to GenBank. Molecular data as well as sequence identity data are summarized in Table 4.

TM was recognized by 68% (21/31) subjects, specific IgE antibodies to *C. crangon* AK were detected in 29% (9/31) of all sera tested. The SCP of *C. crangon* bound IgE antibodies of 35% (11/31) of the subjects, TnC-specific IgE antibodies were detected in 29% (9/31) of subjects. 19% of the subjects reacted to MLC, and TIM was bound by IgE in 23% (7/31) of the cases. Monosensitization to TM occurred frequently, twelve subjects exhibited specific IgE only to this allergen. Three subjects were solely sensitized to SCP and one subject only to TnC. The overall frequencies of IgE antibody binding to the *C. crangon* proteins were considerably lower among the European shrimp-allergic subjects (Table 7) than among the North American shrimp sensitized serum donors (sera A-H, Table 6), who

Table 6
Serum IgE levels and prevalences of IgE binding to single *C. crangon* allergens and shrimp extract for sera of shrimp-sensitized subjects (A-H) as determined by ImmunoCAP analysis.

Serum	ImmunoCAP results [kU_A/L]							
	TM	AK	SCP	TnC	TIM	MLC	Sum of single allergens	Shrimp extract
A	0.60	0.66	6.13	0.59	14.57	31.00	53.55	8.22
B	0.84	0.77	0.77	5.72	0.79	0.74	9.63	3.31
C	0.10	2.14	<0.1	0.17	0.43	<0.1	2.84	5.01
D	13.21	0.90	0.67	0.58	0.96	10.89	27.21	14.80
E	<0.1	0.16	<0.1	<0.1	0.11	0.14	0.41	4.65
F	0.26	0.32	0.20	8.85	0.20	0.28	10.11	2.51
G	35.16	7.22	0.79	7.42	0.97	3.97	55.53	54.10
H	0.82	1.29	0.75	1.89	0.97	1.04	6.76	13.50
Prevalence	7/8 (88%)	8/8 (100%)	6/8 (75%)	7/8 (88%)	8/8 (100%)	7/8 (88%)	8/8 (100%)	8/8 (100%)

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Table 7
Serum IgE levels and prevalences of IgE binding to single *C. crangon* allergens and shrimp extract in shrimp-allergic subjects as determined by ImmunoCAP analysis (positive if IgE concentration ≥ 0.1 kU_A/L).

Serum	ImmunoCAP results [kU_A/L]							
	TM	AK	SCP	TnC	TIM	MLC	Sum of single allergens	shrimp extract
1	72.14	0.14	<0.1	<0.1	0.10	<0.1	72.38	81.50
2	<0.1	<0.1	0.42	<0.1	<0.1	<0.1	0.42	1.45
3	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	–	6.13
4	0.37	0.51	0.84 (1:5)	0.16 (1:5)	0.19 (1:5)	0.16 (1:5)	2.23	3.00
5	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	–	<0.1
6	<0.1	0.76	0.14	0.13	0.23	0.12	1.38	0.50
7	1.63	<0.1	<0.1	<0.1	<0.1	<0.1	1.63	2.55
8	<0.1	<0.1	0.42 (1:5)	<0.1 (1:5)	<0.1 (1:5)	<0.1 (1:5)	0.42	25.30
9	<0.1	<0.1	1.35	<0.1 (1:5)	<0.1 (1:5)	<0.1 (1:5)	1.35	0.66
10	<0.1	<0.1	<0.1	2.72	<0.1	<0.1	2.72	0.76
11	2.10	<0.1	<0.1	<0.1	<0.1	<0.1	2.10	1.04
12	11.78	0.84	0.11	0.45	0.11	<0.1	13.29	16.30
13	8.30	0.12	<0.1	<0.1	<0.1	<0.1	8.42	13.80
14	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	–	1.19
15	>100	2.62	0.14	<0.1	0.12	0.13	>100	>100
16	5.13 (1:2)	<0.1 (1:2)	<0.1 (1:2)	<0.1 (1:2)	<0.1 (1:2)	<0.1 (1:2)	5.13	18.20
17	7.78	<0.1	<0.1	<0.1	<0.1	<0.1	7.78	7.22
18	<0.1	<0.1	<0.1	1.71	0.15	<0.1	1.86	0.25
19	0.33	<0.1	<0.1	0.78	<0.1	<0.1	1.12	0.81
20	<0.1	<0.1	0.15	0.26	<0.1	<0.1	0.41	3.39
21	1.56	12.09	5.45	>100	1.72	4.58	>100	>100
22	1.01	<0.1	<0.1	<0.1	<0.1	<0.1	1.01	1.41
23	0.79	<0.1	<0.1	<0.1	<0.1	<0.1	0.79	0.77
24	0.98	<0.1	<0.1	<0.1	<0.1	<0.1	0.98	1.35
25	7.74	0.37	0.11	2.68	<0.1	2.50	13.40	18.35
26	0.64	<0.1	<0.1	<0.1	<0.1	<0.1	0.64	2.13
27	3.75	<0.1	<0.1	<0.1	<0.1	<0.1	3.75	3.24
28	12.48	<0.1	<0.1	<0.1	<0.1	<0.1	12.48	14.65
29	1.06	<0.1	<0.1	<0.1	<0.1	<0.1	1.06	2.53
30	10.48	<0.1	<0.1	<0.1	<0.1	<0.1	10.48	14.94
31	66.44	0.32	0.11	<0.1	<0.1	0.14	67.01	33.87
Prevalence	21/31(68%)	9/31 (29%)	11/31 (35%)	9/31 (29%)	7/31 (23%)	6/31 (19%)	28/31 (90%)	30/31 (97%)

were only included for the purpose of detection and identification of IgE-binding proteins.

3.6. Single shrimp allergen ImmunoCAP tests show a convincing measuring sensitivity

For quantitative comparison of the measuring performance between extract and single allergen ImmunoCAP tests, IgE values ≥0.1 kU_A/L measured to purified shrimp allergens were summed up as presented in Table 7. These values were compared to IgE levels measured to shrimp extract for each subject. For 16/31 (52%) shrimp-allergic subjects the results were in the same range (serum nos. 1, 4, 5, 7, 12, 13, 15, 17, 21, 22, 23, 24, 25, 27, 28, 30), in 7/31 (23%) the combined single allergen levels were considerably higher (serum nos. 6, 9, 10, 11, 18, 19, 31) and in 8/31 (26%) considerably lower than the IgE concentration to shrimp extract (serum nos. 2, 3, 8, 14, 16, 20, 26, 29). Hence, in 75% of the shrimp-allergic individuals IgE quantification with single allergens was equally or even more sensitive than with shrimp extract ImmunoCAP tests. The same was observed for the subjects with sensitization but unknown allergy to shrimp (see Table 6) with 50% of these sera containing considerably higher IgE levels to the purified allergens than to the extract.

3.7. Diagnostic sensitivity of single shrimp allergen ImmunoCAP tests is 90%

The qualitative comparison of IgE antibody reactivities to single allergens and to shrimp extract by ImmunoCAP analysis showed that specific IgE to shrimp extract was detected in 30/31 (97%) of the subjects whereas 28/31 (90%) sera recognized at least one of the recombinant *C. crangon* proteins. Thus, the diagnostic sensitivity of a combination of the single allergen components was only slightly below that of shrimp extract. Subject no. 5, who did not show IgE binding to shrimp extract did also not have detectable IgE to any of the single *C. crangon* allergens. Sera of two subjects (nos. 3 and 14) with IgE specific to shrimp extract did not react to any of the single allergens studied. These two sera contained extract-specific IgE levels that were considerably above the detection limit (1.19 and 6.13 kU_A/L) and may define one or more shrimp allergens yet to be identified.

4. Discussion

Knowledge about the allergen profile of crustaceans is still limited. Immunoblotting analysis showed a large number of IgE-binding proteins. However at the time we initiated this study only two allergens, tropomyosin and arginine kinase, were known. Meanwhile, a parallel study reported the identification of sarcoplasmic calcium-binding protein and a myosin light chain (MLC) as shrimp allergens (Ayuso et al., 2008, 2009; Shiomi et al., 2008). However, our data presented here indicate that these four allergen families do not fully represent the IgE-binding capacity of shrimp extract. As detailed knowledge about the allergen components is essential to develop sensitive diagnostic and therapeutic reagents that are well defined at the molecular level it was our aim to generate a panel of allergens accounting for the full spectrum of IgE responses to shrimp.

In this study we generated a comprehensive panel of recombinant shrimp allergens, comprising six allergens by a combination of proteomic and molecular biologic methods. The recombinant shrimp proteins were evaluated for IgE reactivity among sera of 31 European shrimp-allergic subjects, who predominantly originated

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from Central Europe. According to ImmunoCAP analysis, all six proteins, TM (Cra c 1), AK (Cra c 2), SCP (Cra c 4), MLC (Cra c 5), troponin C (TnC, Cra c 6) and triosephosphate isomerase (TIM, Cra c 8) proved to be allergens. TnC and TIM are reported as crustacean allergens for the first time and the MLC identified and cloned in this study was a variant entirely different from the white Pacific shrimp allergen Lit v 3. All six proteins are abundant in shrimp muscle and are involved in muscle contraction or energy metabolism.

Cra c 1 joins the group of well-characterized tropomyosins which have been described as allergens in various invertebrates. Sequence identities between crustacean TMs are very high, e.g. Cra c 1 shares more than 90% amino acid identity with different shrimp and lobster TMs. This protein family has been described as a main cross-reactive structure among invertebrate species (reviewed by Lopata and Lehrer, 2009).

Cra c 2 is the third confirmed IgE-binding crustacean arginine kinase. Previously reported allergenic AKs are Lit v 2 from *L. vannamei* (Garcia-Orozco et al., 2007), accession no. ABI98020) and Pen m 2 from black tiger shrimp *Penaeus monodon* ((Yu et al., 2003), accession no. AAO15713) both of which share 93% sequence identity with Cra c 2.

Cra c 4 is a member of the recently identified allergen class of sarcoplasmic Ca-binding proteins. IgE-binding homologues were described for *L. vannamei* (Lit v 4 (Ayuso et al., 2009)) and *P. monodon* (Pen m 4 (Shiomi et al., 2008)). In our study three patients were exclusively sensitized to SCP.

Cra c 5 represents a novel type of IgE-binding myosin light chains. MLCs were already described as IgE binding in *L. vannamei* (Lit v 3 (Ayuso et al., 2008)) and in cockroach (Bla g 8, (Yong and Jeong, 2009)) but the sequence identity between Cra c 5 and Bla g 8 (accession no. ABD47458) or Lit v 3 (accession no. ACC76803) is only 13%. This points towards an independent novel group of allergenic myosin light chains. Apparently, several MLC molecules seem to be involved in IgE binding.

Cra c 6 is the first crustacean troponin C to which IgE binding is reported. TnC has previously been identified as an IgE binding protein in cockroach (Bla g 6 (Hindley et al., 2006)).

Cra c 8 is another novel shrimp allergen. While triosephosphate isomerase has not before been identified as an allergen in crustaceans, representatives of this protein family have been reported as IgE-binding proteins in fish, midges and various plants (Chen et al., 2005; Hoppe et al., 2006; Nakamura et al., 2009; Pastor et al., 2009; Posch et al., 1997; Sander et al., 2001).

Cra c 1 was the only major allergen in this study and bound IgE from 68% of the shrimp-allergic subjects of our study. All other allergens showed IgE binding in 19–35% of the subjects, rendering them a classification as minor allergens. Even though tropomyosin has been described as the dominant crustacean allergen (Daul et al., 1994), the present study for the first time brings together a comprehensive panel of crustacean allergens and demonstrates that Cra c 6 (TnC), Cra c 4 (SCP) and Cra c 2 (AK) bound more IgE than Cra c 1 (TM) in approximately one third of the cases. The importance of Cra c 6 (TnC), Cra c 4 (SCP) and Cra c 2 (AK) as diagnostically relevant allergens was further highlighted by the observation that 7 of 10 sera that did not react with Cra c 1 (TM) showed IgE binding either to Cra c 2 (AK), Cra c 4 (SCP) or Cra c 6 (TnC). IgE reactivities to Cra c 8 (TIM) or Cra c 5 (MLC) were lower than those to Cra c 2 (AK), Cra c 4 (SCP) or Cra c 6 (TnC), further indicating an importance of the latter three allergens in particular. However, as some sera had to be diluted prior to the ImmunoCAP analysis of IgE binding to the recombinant *C. crangon* allergens (indicated in Table 7), the true frequency of sensitization to Cra c 5, Cra c 6 and Cra c 8 may have been somewhat underestimated.

IgE-binding frequencies in ImmunoCAP analysis were much higher for individuals with shrimp-sensitization but unknown clinical history (Table 6) than for those with a confirmed shrimp allergy (Table 7). This might be due either to a different geographical background of these subjects (USA vs. Europe), to differences in their clinical status (sensitized vs. allergic), or to a primary, cross-reactive sensitizer other than shrimp. These results highlight that a well chosen patient panel is very important for the outcome of a study on allergens and confirm the need for future CRD studies that compare clinically well characterized patients groups with different geographic or clinical background.

The performance of the single allergen ImmunoCAP tests was analyzed with regard to measuring sensitivity. For this purpose, the sum of IgE values measured for the single allergens were compared to the IgE levels to shrimp extract. For half of the subjects the values were comparable, meaning that IgE binding to the single allergens largely accounted for the IgE binding to the natural shrimp extract (e.g. patients 12 or 17). For about one quarter of the subjects, the sensitivity of the measurement was higher with the single allergen components, probably because certain allergens are underrepresented in the extract (e.g. TnC for patients 10 or 19). For about 75% of both, the shrimp-allergic and the sensitized subjects, the IgE levels to the single allergens were similar or even higher than IgE values to the extract. This demonstrates the potential of the single allergen components as sensitive diagnostic tools. However, for another quarter of the allergic subjects, the IgE levels measured using shrimp extract as test antigen considerably exceeded the sum of the values measured to the single allergens. This may be due to failure of the recombinant proteins to fully reproduce the IgE-binding determinants of the corresponding natural proteins, even if we have observed no indication to that effect. Alternatively, we may have missed yet unidentified allergens in the extract because additional IgE binding to proteins of higher molecular weight than investigated was seen in very few subjects. Moreover, one candidate molecule might be a myosin light chain variant of the kind represented by Bla g 8 and Lit v 3 that we did not find.

Even though there might still be allergens missing in the current panel, we were able to detect shrimp-specific serum IgE in 28/31 (90%) subjects by testing the three novel in addition to three known shrimp allergens in a single component ImmunoCAP analysis. Consequently, IgE reactivities could be detected in almost any studied shrimp-allergic individual, and the single allergen components were basically capable to mimic the allergenic complexity of the extract that bound IgE from 30/31 (97%) sera. In addition to verification of a general sensitization to shrimp by already existing and well performing extract reagent, this extensive panel of shrimp allergens provides a valuable tool for future component resolved diagnosis studies with patients from different geographic areas. As one result, different aspects of clinical relevance of the single allergens may be assessed and the novel allergens might serve as targets for the development of new therapeutic approaches of shrimp allergy. Furthermore, by comparing sensitization patterns from patients with different symptoms or severity of symptoms, clinically useful biomarkers might be identified.

We used the shrimp species *C. crangon* (North Sea Shrimp) as a European model shrimp for this study, since a previous report (van Ree et al., 1996) indicated this species as allergenic and because of its commercial importance. *C. crangon* is found in most European seas and has a significant commercial relevance with landings of approximately 40,000 tons in 2007 (ICES Living Resources Committee, 2007). Furthermore, as previously reviewed by Sicherer (Sicherer, 2001) the degree of clinical cross-reactivity among crustacean species is high and clinicians advise patients who are allergic to one species to avoid any crustacean. As a consequence, the three novel allergens identified from this source material are very likely representative for allergens of a variety of other frequently consumed shrimp species.

In summary we identified six allergens from the North Sea Shrimp *C. crangon*, three of which are novel, that may contribute

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to the development of more sophisticated and clinically adequate methods of diagnosis and treatment of shrimp allergy.

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